Synopsis

Alleviating effect of *Rosa indica*petal extracts on heat stress induced male infertility

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Introduction

Infertility is defined as failure to achieve a clinical pregnancy after 12 months of regular unprotected intercourse. Infertility rates have dramatically increased in the last two decades, especially in men resulting from various pre-testicular, testicular and post-testicular causes, of which heat stress is one of the common cause for male infertility.

According to a latest research data from World Health Organization (WHO) carried out over a period of 20 years, infertility affects approximately 48.5 million couples worldwide of which 40% is contributed by male factor.

According to a recent study, fertility rates in men younger than 30 years has decreased by 15% due to obesity, occupational exposure, exercise, diet, electronic devices, stress, scrotal temperature, ROS, Therapeutic drugs etc.

The present study focus on the increase in scrotal temperature with long hours of sitting, which impairs the ability of scrotum to thermo regulate, with an adverse effect on quality and quantity of sperm produced and the alleviating effect of *Rosa indica* petal extracts on steroidogenesis and spermatogenesis and its outcome on sperm quality in heat stress induced male infertility

Medicinal plants have been the basis of treatment for a long period in Asian and African folk medicine. They are the natural resources of modern medicine since they are the potential source of phytochemical compounds like steroids, terpenoids, glycosides, glycoprotein, proteins, alkaloids, flavonoids etc[1]. Phytochemicals have vastly been studied for their antioxidant, antitumor, immuno-modulatory, antimicrobial properties [2]. Many plants have been studied for their male fertility-enhancing properties. *Eurycoma longifolia* jack referred to as Tongkat, a native of East Asia has found to be rich in various phytochemicals and have been indicated for a wide range of activities such as anti-malarial, anticancer and antibacterial besides treating male infertility [3]. The effect of ethanolic extract was studied on both androgenic and proliferative activity and was found to overcome the late-onset of hypogonadism by biosynthesis of various androgens [4]. *Cardiospermum halicacabum* is a popular herb of Srilanka commonly referred to as balloon vine. Various doses (200, 400 and 800 mg/kg, twice daily for ten days) have found to increase caput and epididymal sperm count in rats due to its rich phytocompounds especially

flavanoids [5]. Grape seed extract has shown to increase semen parameters including sperm count, viability and motility in a study on aluminium chloride-induced testicular dysfunction in rats and has been reported to reduce the germ cell apoptosis induced by testicular torsion [6]. *Syzygium aromaticum*, a native of Indonesia, commonly referred to as clove, has traditionally been used as a cure for sexual dysfunction and libido [7]. Streptozotocin-induced diabetic rats were treated with 0.5ml/day for two months with *Nigella sativa*, which are rich in more than hundred phytocompounds and has shown to increase testosterone [8]. Normal and hemicastrated rats were treated for 14 and 21 days with 10 mg/kg of *Lycium barbarum* a Chinese herb which has long been used as an aphrodisiac and has proved to attenuate the testicular DNA damage and also by increasing testosterone [9]. *Tribulus terrestis* a perennial creeping herb has been identified as a cure for treating male infertility in Asia and Europe. A study of *Tribulus terrestis* on sodium valproate treated male rats showed a dose-dependent increase in the level of Testosterone (T), Follicule stimulating hormone (FSH) and Lutenising hormone (LH) [10]. *Asteracantha longifolia* have potentially been used as an aphrodisiac and androgenic agent for centuries. Ethanolic extract of *Polycarpea corymbosa* a local herb of Tamil Nadu increased the level of T and LH [11]. A study conducted on mouse Leydig cells reported the steroidogenic effect of *Taraxacum officinale* extract [12]. *Rosa damascena*, an ancient holy herb, was studied for its androgenic effect on male Wistar rats. 400mg/kg of the of the extract for 21 days found to increase FSH, LH and T.

Rosa Indica is a perennial flower shrub of the genus Rosa. It belong to family Rosaceae, which contains herbs, shrubs or trees that are rhizomatous, thorny or climbing (13,14). They are sharply toothed oval shaped leaflets. The fruit of plant is fleshly, edible (rose hip) which ripens in late summer. Roses are rich source of vitamin c and are used for making various herbal preparations (15). Ethnopharmocologically, roses have been used to treat various eye diseases and heart diseases (16). In the Indian system of medicine, various rose preparations are used as astringent, tonic, mild laxative, antibacterial agent, in treating sore throat, enlarged tonsils, gall stones and also a vehicle for the preparation of other medicines (17). A study in 2017 reported the antimicrobial effect of ethanolic extract of *Rosa indica* on *E.coli, S.aureus, Salmonella sp, K.pneumonia* and *Vibrio sp* (18). However, there is a lack of study pertaining the medicinal uses of various extracts of *R.indica* on male infertility. Considering these informations, this study explores the alleviating effect of *R indica* on heat stress induced male infertility.

Objectives

1. To study the impact of environmental factors and life style habits on semen parameters
2. To study the *Invitro* antioxidant and anti-inflammatory activity of various dry and fresh Rosa indica petal extracts
3. To study the alleviating effect of various dry and fresh *Rosa indica* petals extracts on steroidogenesis and spermogenesis on heat stress induced male wistar rats

Phases of the study

Phase I - To study the significance of environmental factors and life style habits on semen parameters

Phase II – Phytochemical analysis of various extracts of fresh and dry *Rosa indica* petals

Phase III – *Invitro* anti-oxidant and anti-inflammatory properties of various extracts of dry and fresh *Rosa indica* petals and its cytoprotectivity on TM3 cell lines

Phase IV – The alleviating effect of dry extracts of *Rosa indica* petals on steroidogenesis and spermatogenesis and its outcome on sperm quality in heat stress induced male wistar rats

Inclusion criteria

a) Age between 25 yrs to 45 ys
b) Sub fertility
c) Primary and secondary infertility
d) Female factors- age, uterine anomalies, tubal factor, hormonal factors etc

Exclusion criteria

a) Age above 45 yrs
b) Any other illness including Diabetes mellitus
c) Neurological problems
d) Corrective surgeries for reproductive anomalies
Scrotal and spine injuries
Retrograde ejaculation

Methodology and Outcome of the study

Phase I

A retrospective data of environmental factors and life style parameters was studied from 299 patients consulting for infertility and sub fertility to a private hospital in Coimbatore using questionnaire and correlated with semen parameters as per the WHO guidance (2010) after obtaining Human ethics clearance (AUW/IHEC/BCBT-19-20/XPD-01).

The questionnaire included: age, sexual abstinence, season of sample collection, addictions (alcohol consumption, smoking), nature of job (sitting hours).The Semen parameters studied were appearance, viscosity, volume, pH, sperm concentration, progressive motility and sperm morphology.

A binary logistic regression analysis was performed with result (normal or abnormal) semen report as dependent variable, age, abstinence days as covariate and season, sitting, alcohol, smoking and tobacco as independent variables. It was noted that abstinence days has significant effect in predicting abnormality. As the abstinence days increased, there was an improvement in semen parameters studied.

Linear regression analysis with fixed intercept model was performed with sperm concentration as dependent variable, age, abstinence days as covariate, season, sitting, alcohol, smoking and tobacco as independent variables. It was noted that season had significant effect in predicting sperm concentration. Season 3 and 4 (july- December) had less sperm concentration as compared season 1 (January- march). Abstinence days had significant effect in estimating sperm concentration. There was an increase in sperm concentration with increase in abstinence days. Sitting had significant effect on sperm concentration. The longer hours the person sits the concentration reduced by 9 units.

Linear regression analysis with fixed intercept model was performed with motility as dependent variable, age, abstinence days as covariate, season, sitting, alcohol, smoking and tobacco as independent variables. It was noted that abstinence days had significant effect in estimating sperm motility. There was an increase in motility with increase in abstinence days. Sitting had significant effect on sperm motility. The longer hours the person sits the percentage motility reduced.

Linear regression analysis with fixed intercept model was performed with normal forms as dependent variable, age, abstinence days as covariate, season, sitting, alcohol, smoking and tobacco as independent variables. Season had significant effect on normal forms. Season 3 and 4 (July-December) had less sperm concentration as compared season 1 (January-March). Abstinence days had significant effect on normal forms. There was an increase in normal forms with increase in abstinence days.

With this result the further study was proceeded to find the alleviating effect of various extracts of *Rosa indica* on heat stress induced male infertility.

Phase II

1. Preparation of Dry and Fresh R.indica petal extracts

Rosa indica petal extracts were prepared using the solvents including Double distilled water, 100% Ethanol and Chloroform using Cold extraction method with an intension not to lose the volatile oils. The extracts were subjected to **preliminary phytochemical screening**. The Dry and fresh aqueous and ethanolic extracts which were found to be rich in phytocompounds where further subjected to **quantification** according to standard protocols with slight modifications. Chloroform extract which showed only the presence of saponins was not included for further studies. Fresh ethanolic extracts exhibited highest concentrations of flavanoids, dry ethanolic extract showed highest concentration of alkaloids and phenols where as dry aqueous extract showed highest concentration of Phytosterols. Presence of secondary metabolites including alkaloids, flavonoids, terpenoids, phenols and coumarins were further confirmed by **HPTLC** and the chemical compounds present in various extracts were analysed using **GCMS**. Kaempherol a natural flavonol, a type of flavanoid was present in all the extracts. In addition to kempferol, Alpha tocopherol or Vitamin E which has a major role in combating Reactive oxygen species in spermatozoa and linolenate, a Poly Unsaturated Fatty Acid having major role in steroidogenesis, andrographolide which help to reduce reactive oxygen species and increase testosterone were found to be present in ethanol dry extract.

Phase III: *Invitro* anti-oxidant, anti-inflammatory and Cytoprotectivity study of Rosa indica petal extracts

1. 20-100 µl of 1mg/ml concentration of the extracts were studied for their ***Invitro* antioxidant properties** including DPPH, ABTS, Nitric oxide radical scavenging, Hydroxyl radical scavenging, Reducing power assay and Hydrogen peroxide radical scavenging according to standard protocols. Highest concentration of aqueous dry and ethanolic fresh extract showed

highest % inhibition of DPPH at 518 nm respectively. Highest concentration of ethanolic dry and aqueous fresh extract showed the highest % scavenging for ABTS at 745 nm. Highest concentration of ethanol dry extract showed the highest Nitric oxide radical Scavenging of at 546 nm. Ethanolic dry extract showed the highest % inhibition of hydroxyl radical scavenging property at 532 nm. Ethanolic dry extract showed highest reducing power of at 700 nm. Ethanolic dry extract showed the highest % inbition for hydrogen peroxide radical scavenging at 532nm.

2. **Anti-inflammatory property** was studied using Egg Albumin Denaturation assay according to Mizushima et al., 1968. Ethanolic dry extract exhibited the highest invitro anti-inflammatory activity .

3. TM3 cell lines (Mouse leydig cells) derived from 11-13 d mouse testes was used to study the lethal dose of various extracts of *R indica* petals using **MTT assay**. DMEM supplemented with 2% fetal bovine serum was used as the nutrient media at 37 ^{o} C with 5% CO_2. 5000 cells/ 200 µl/well were seeded in 96 well plate for 24 and 48 hrs with varying concentrations of the extracts 50- 250 µg. From the cell viability assay it could be inferred that both aqueous and ethanol dry and fresh extracts of *Rosa indica* were efficient in maintaining the highest % viability of TM3 cell lines even at the highest concentration of 250 µg studied after 24 hrs and 48 hrs.

In view of the high efficacy of the dry extracts, (200 mg/kg body weight of rat) aqueous dry : aqueous fresh and ethanol dry : ethanol fresh extracts as 1: 1 ratio of *Rosa indica* petals were further studied for their alleviating effects on steroidogenesis and spermatogenesis and its outcome on sperm quality in heat stress induced male wistar rats.

Phase IV

After obtaining animal ethics clearance with approval number (COPSRIPMS/IAEC/PHD/PROJECT/001/2020-2021), the study was conducted on 3 months old male wistar rats weighing 180-240 g. The animals were grouped into 5, each group containing 6 animals (total of 30 animals). Study duration was totally 28 days of which 07 days for acclimatization and 21 days for study. Heat stress was induced by immersing hindlegs, tail and scrotum in 43°C thermostatically controlled water bath for 30 min for 6 consecutive days from day 8 of the treatment.

	Groups	**Route of Administration**
Group 1	Control (feed and water only)	-----
Group 2 (Negative control)	Heat stress	-----
Group 3 (Positive control)	Heat stress + 50 mg/kg quercetin in 0.5% carboxy methyl cellulose	Intragastric Gavage
Group 4	Heat stress + 200 mg/kg of ethanolic extract (dry:fresh, 1:1) in 0.5% carboxy methyl cellulose	Intragastric Gavage
Group 5	Heat stress + 200 mg/kg of aqueous extract (dry:fresh, 1:1) in 0.5% carboxy methyl cellulose	Intragastric Gavage

Body weight of the animals were recorded on the day of receiving and on 28th day of the study. Though there was a clinical significance, there was no statistical significance in weight of animals in different groups before heat treatment. Whereas there was a decrease in body weight of animals in negative control and an increase in body weight of animals in positive control after heat treatment.

After sacrificing the animal under ketamine I.P 100 mg/kg on 28th day the following tests were performed.

1. Blood was drawn by cardiac puncture , centrifuged at 3000 g (10 min, 4^{o}C) to obtain serum for the estimation of **Serum Testosterone** (T) using ELICA kit specific for rats from ROCHE. ethanolic extract showed highest concentration of testosterone which was comparable with positive control, where as negative control group showed the lowest concentration of testosterone. This shows the effect of heat stress on steroidogenesis and the alleviating effect of the ethanolic extract on heat stress induced impairment of steroidogenesis.

2. **Testis** was removed and weighed, negative control had the lower testis weight compared to the other groups with a significant $P<0.004$.

3. Cauda epididymis was dissected and minced in MOPs buffer to release the sperm to carry out further studies.

4. **Semen parameters** including sperm Concentration (M/ml), progressive motility (%) and normal forms (%) were analyzed using inverted microscope from the tissue extracted from the cauda epididymis. The animal group treated with ethanolic extract exhibited comparably good semen parameters to positive control, where as the animal group treated only with heat stress had lowest studied parameters with a significant value of $p< 0.05$

5. One testis stored in buffered 10% Formalin for **Histopathology** was processed according to the previous studies and viewed under 10X and 40 X objective of light microscope. Control group showed normal spermatogenesis with normal sertoli cells, leydig cells and seminiferous tubules. Negative control group showed germ cell aplasia, mild hyperplasia of leydig cells and maturation arrest. Animals in positive control showed occasional leydig cells with normal spermatogenesis in upto 90% of the seminiferus. Heat stress group treated with ethanolic extract showed normal spermatogenesis in upto 80% of the tubule with normal sertoli cells and leydig cells. Heat stress group treated with aqueous extract showed normal spermatogenesis in upto 50% of the tubule with sparsely normal sertoli cells and leydig cells .

6. Other testis was used for performing ***Invivo*** **antioxidant assays** including glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase and lipid peroxidation was performed according to standard procedures with slight modifications. All the values are expressed as mean ± standard deviation, n= 6.

Glutathione peroxidase (Paglia DE, 1967) was found to be highest in ethanolic extract treated group comparable to control group and lowest in aqueous extract group

Glutathione reductase (Racker E, 1955) was found to be highest in ethanolic extract treated group comparable to control and lowest in negative control.

Superoxide dismutase (Kakkar P, 1984) was highest in the control group followed by ethanolic extract treated group and lowest in negative control

Catalase (Sinha AK, 1972) was found to be highest in ethanolic extract treated group comparable to positive control and lowest in negative control

Lipid peroxidation (Ohkawa H, 1979) was found to be highest in negative control and lowest in aqueous extract treated group.

Outcome of the study

From the study, it could be confirmed that heat stress drastically affects the testis, lowers the level of testosterone, spermatogenesis and thus the semen quality, which is alleviated by the sequestering effect of alpha tocopherol, linolenate and andrographolide in addition to kempferol and other phytocompounds in ethanolic extract.

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